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Segregation of lens and olfactory precursors from a common territory: cell sorting and reciprocity of Dlx5 and Pax6 expression ☆

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Abstract

Cranial placodes are focal regions of columnar epithelium next to the neural tube that contribute to sensory ganglia and organs in the vertebrate head, including the olfactory epithelium and the crystalline lens of the eye. Using focal dye labelling within the presumptive placode domain, we show that lens and nasal precursors arise from a common territory surrounding the anterior neural plate. They then segregate over time and converge to their final positions in discrete placodes by apparently directed movements. Since these events closely parallel the separation of eye and antennal primordia (containing olfactory sensory cells) from a common imaginal disc in *Drosophila*, we investigated whether the vertebrate homologues of Distalless (Dll) and Eyeless (Ey), which determine antennal and eye identity in the fly, play a role in segregation of lens and nasal precursors in the chick. *Dlx5* and *Pax6* are initially co-expressed by future lens and olfactory cells. As soon as presumptive lens cells acquire columnar morphology all Dlx family members are down-regulated in the placode, while Pax6 is lost in the olfactory region. Lens precursor cells that express ectopic Dlx5 never acquire lens-specific gene expression and are excluded from the lens placode to cluster in the head ectoderm. These results suggest that the loss of Dlx5 is required for cells to adopt a lens fate and that the balance of Pax6 and Dlx expression regulates cell sorting into appropriate placodal domains.

Keywords: Chick; Distal-less; Dlx; Eye; Eyeless; Fate map; Nasal epithelium; Pax6; Placode; Sensory organ

Introduction

In the vertebrate head, critical parts of the peripheral sensory nervous system arise from transient ectodermal thickenings, the cranial placodes, which develop at unique positions next to the neural tube (Baker and Bronner-Fraser, 2001). The olfactory placode gives rise to the nasal epithelium, the lens placode to the crystalline lens of the eye and the otic placode forms the inner ear. The trigeminal and the three epibranchial placodes, together with neural crest cells, form the cranial ganglia.

In the chick embryo, cells that will contribute to different placodes (like future otic and epibranchial cells) are initially intermingled and only later segregate to form separate placodes (Streit, 2002). Like otic precursors in the chick, olfactory cells in zebrafish are recruited from a large, but defined region of the head ectoderm and converge to their final position through cell rearrangements and movements (Whitlock and Westerfield, 2000). These observations raise the possibility that an initial step in placode formation is the establishment of a pre-placodal domain containing precursors for multiple placodes and that unique regional identities are imparted as a later step. In agreement with this notion, classical embryological experiments indicate that common tissue interactions and probably common signals are required for the initial induction of different placodes (Jacobson, 1963a,b,c). Moreover, the paired-domain transcription factor *Pax6* (Walther and Gruss, 1991) as well as members of the Six (Bovolenta et al., 1998; Esteve and Bovolenta, 1999; Oliver et al., 1995; Pandur and Moody, 2000) and Eya (Mishima and Tomarev, 1998; Sahly et al., 1999; Xu et al., 1997) families are expressed in both nasal and lens placodes, and loss of Pax6 function results in the failure of both of these placodes to form (Grindley et al., 1995;

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Quinn et al., 1996; van Heyningen and Williamson, 2002; Wawersik et al., 2000).

The idea that olfactory and visual cells may share a common origin is surprisingly reminiscent of the development of sensory organs in holometabolous insects: the antenna, an odour-detecting organ, and the eye arise from a common imaginal disc, the eye-antenna disc. During larval development, these territories separate and acquire distinct identities to give rise to the adult antenna and compound eye. This process is partially dependent on the action of two transcription factors that seem to regulate each other (Kurata et al., 2000): the Dlx gene Distalless (Dll) is required to confer antennal identity (Cohen et al., 1989; Dong et al., 2000; Panganiban and Rubenstein, 2002; Sunkel and Whittle, 1987), while the Pax6 homologue Eyeless (Ey) is essential for eye specification (Halder et al., 1995; Quiring et al., 1994; for review: Gehring, 1996; Kumar and Moses, 2001c).

This raises the intriguing question of whether a similar principle may hold true for vertebrate nasal and lens placode formation. Here, we show in the chick that precursors for these two placodes arise from a common territory next to the anterior neural plate and segregate over time by apparently directional movements. As in the fly, *Dlx5* and *Pax6* are initially co-expressed in the common nasal-lens domain. As streams of cells destined to the lens and to olfactory regions segregate, expression of these two transcripts separates accordingly, suggesting that cell migration and regulation of these genes are coordinately regulated. However, the proteins they encode only become differentially expressed as the placodes begin to form: Dlx5 expression is lost from the lens and Pax6 expression is transiently down-regulated in nasal precursors. Loss of Dlx5 is required for cells to acquire a lens identity: no cells that continued to express Dlx5 were found in the lens. This points to a remarkable and hitherto unnoticed similarity in the developmental processes that generate olfactory and visual organs in vertebrates and arthropods.

Materials and methods

Embryo techniques

Fertile hens' eggs (Winter Farm, Hertfordshire, UK; Spafas, Charles River Laboratories, Roanoke, IL, USA) were incubated at 38°C for 24–45 h to obtain embryos at stages 6–10 (Hamburger and Hamilton, 1951). For fate mapping, small groups of epiblast cells were labelled using the fluorescent dyes DiI and/or DiO as described previously (Ruiz i Altaba et al., 1993). Briefly, stocks of 0.5% DiI or of 0.25% DiO in absolute alcohol or DMSO were diluted 1:10 in 0.3 M sucrose at 50°C and injected by air pressure using a micropipette made from 50 μl borosilicate glass capillaries. The labelled position was measured in relation to other landmarks (see below) and the embryos then cultured in ovo until the lens and olfactory placodes could be identified by morphological criteria (stages 15–19). The position of labelled cells was assessed in whole mounts or after cryosectioning.

For video time-lapse analysis, embryos were labelled as described above, incubated for 1–2 h in ovo and then explanted dorsal side down on fibronectin (20 μg/ml)-coated Millicell inserts (Millipore) and cultured in Neurobasal medium containing B27 supplement as previously described (Krull and Kulesa, 1998).

Standardisation of the position of labelled cells

The anteroposterior and mediolateral positions of DiI- and/or DiO-labelled cells were measured using an eyepiece graticule immediately after injection. In stage 6–7 embryos, the distances from the centre of Hensen's node (primitive pit) to the tip of the prechordal plate (pp-hn = 100%; Fig. 1A) and to the labelled cells, respectively, were measured and the position of the label was calculated as a percentage of the total length pp-hn. To standardise the mediolateral position, the distance between the midline and the labelled cells was expressed as percentage of the distance between the midline and the edge of the neural plate at the level just anterior to the node (ml-np = 100%; Fig. 1A).

In embryos with two to five somites, distances were measured from the anterior edge of the first somite to the anterior neural ridge (anr-som = 100%; see Fig. 1B) and to the labelled cells. The position of the label was calculated as percentage of the total distance anr-som. The mediolateral position of the labelled cells was determined in relation to the width of half the neural plate at the level just anterior to the first somite (ml-np = 100%; see above and Fig. 1B).

In embryos older than five somites, the distances from the first somite to the neuropore (np-som = 100%; Fig. 1C) and to the labelled cells, respectively, was measured and the position of the label expressed as percentage of the total length np-som. To standardise the mediolateral position, the distance from the midline to the labelled cells was expressed as percentage of the distance between the midline and the lateral edge of the optic vesicles (ml-ov = 100%).

Note that using this system, these measurements are relative such that the 100% value differs considerably between the mediolateral and anteroposterior axes, as well as between different stages.

Video time-lapse filming

Four different positions of the epiblast on each side of stage 7–8 embryos were labelled with DiI as described above. The embryos were cultured in a heated chamber placed around an inverted Zeiss 410 laser scanning confocal microscope. For some movies, 3D stacks of pictures were taken every 10–15 min. These stacks were of 70 μm in thickness, with individual sections 14 μm apart. In other cases, the pinhole was opened up completely and a single thick section

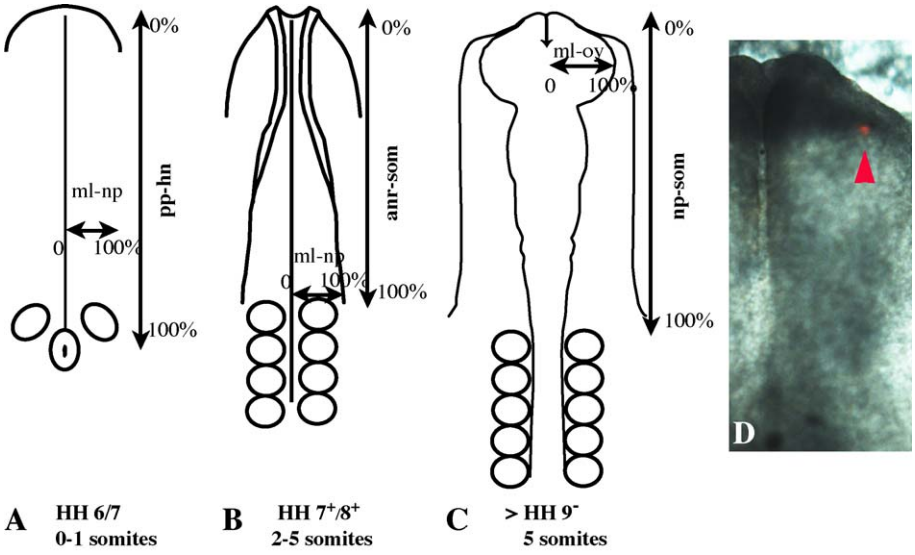

Fig. 1. Diagram showing the standardisation of the injection sites relative to other landmarks. (A) In stage HH6/7 embryos (0–1 somites), the distance between the centre of Hensen's node and the anterior tip of the prechordal mesoderm (pp-hn; 0–100%) was measured and set to 100%. The position of labelled cells along the anteroposterior axis was expressed as % pp-hn. The mediolateral position was expressed as percentage of the distance between the midline (0%) and the lateral edge of the neural plate (100%; ml-np; half the width of the neural plate). (B) In embryos with two to five somites ($HH7^{+}$–8^{+}), the mediolateral position of the labelled cells was determined as described in A. The distance from the anterior neural ridge to the anterior border of the first somite was set to 100% (anr-som) and the anteroposterior position of the labelled cells was expressed as % anr-som. (C) In embryos with more than five somites (>$HH9^{-}$), the anteroposterior position of labelled cells was measured as described in B. Their mediolateral position was determined as percentage of the distance between the midline and the most lateral edge of the optic vesicle (ml-ov). (D) Example of an embryo labelled with a single DiI injection at HH7 (one somite stage).

was imaged at an interval of 5–7 min. All movies were filmed at 5× or 10× magnification. Cell migration was visualised using Quick Time and groups of cells were tracked using Image J. To determine the trajectories taken by the labelled cells in an unbiased manner, all the time frames for each movie were collapsed into a single image. Single channel information at successive time points was opened as an image sequence in Image J and a Z projection, at maximum intensity, of the resulting stack was created.

Whole mount in situ hybridisation, immunohistochemistry and histology

cDNAs for Dlx-5 (Ferrari et al., 1995) and Pax6 (Goulding et al., 1993) were kindly provided by R.A. Kosher and A. Bang. Whole mount in situ hybridisation using DIG-labelled antisense RNA-probes was performed as previously described (Streit et al., 1997; Thery and Stern, 1996). The colour reaction was developed using NBT/BCIP as a substrate. After postfixing, the embryos were embedded in ovalbumin/agar for vibratome sectioning. DiI- and DiO-labelled embryos were embedded in gelatin and 10-μm cryosections were cut.

Mouse monoclonal antibody against Pax6 was obtained from Developmental Studies Hybridoma Bank (Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, and Department of Biological Sciences, University of Iowa, Iowa City 52242, under contract N01-HD-2-3144 from NICHD); polyclonal antibodies recognising all Dlx proteins were a kind gift from Jhumku Kohtz, Northwestern University; polyclonal antibodies against chick δ-crystallin were generously provided by Joram Piatigorski, National Eye Institute. Rabbit anti-GFP antibodies were purchased from Molecular Probes. For immunohistochemistry, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 1 h and embedded in gelatin for cryosectioning. Immunostaining was performed as described previously (Stern, 1993) using anti-rabbit, -mouse or -sheep secondary antibodies coupled to Alexa fluor 488 and 594 (Molecular Probes). Nuclei were visualised with DAPI (Molecular Probes).

Expression constructs and in ovo electroporation

The coding sequence of chick Dlx5 was cloned into pCAB-IRES-eGFP as previously described (McLarren et al., 2003) to generate a bicistronic expression construct under the control of the ubiquitous chick β actin promoter. pCAB-IRES-eGFP without insert was used as control.

Exogenous DNA (2–5 μg/μl) was injected in ovo under the vitelline membrane overlying the presumptive nasal-lens ectoderm of embryos between stage HH8 and 10. DNA transfer into the ectoderm was achieved by electroporation using one broad silver (cathode) and one pointed tungsten (anode) electrode to apply four pulses of 20 V, 50 ms at 1000-ms intervals. Eggs were then sealed and incubated for 1–2 days until the embryos had reached HH13–20. Specimens were recovered in PBS, photographed and processed for cryosections and immunohistochemistry.

Results

In the chick, the lens and olfactory placodes are first visible as patches of thickened epithelium at the 12–14 (HH11) and 21–23 somite stage (HH14), respectively (Bancroft and Bellairs, 1977; Romanoff, 1960). To investigate whether precursors for both placodes arise from a common territory, we constructed fate maps at different developmental stages. Small cell populations in the epiblast of chick embryos from head fold (HH6) to the 12-somite stage (HH11) were labelled with the fluorescent dyes DiI and DiO. Their position in relation to other landmarks was measured immediately after labelling (Figs. 1, 2A″–F″). One dye injection on average labelled 10–30 cells. Embryos were allowed to develop until stage HH15–18, when both placodes are morphologically visible, and the position of the labelled cells was determined in whole mounts (Figs. 2A–F). Some of the embryos were then sectioned to confirm the location of labelled cells (Figs. 2A′–F′). In total, 429 embryos were labelled; of the 325 survivors, most had received one DiI and one DiO injection on each side of the embryo.

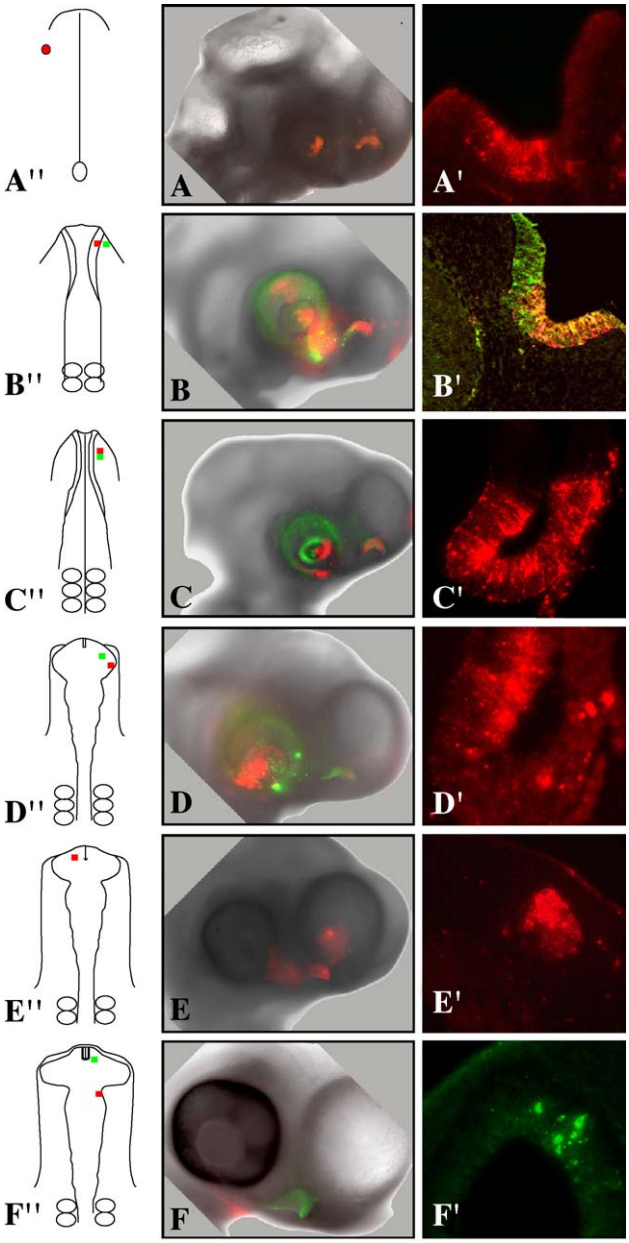

Fig. 2. Examples of DiI- and DiO-labelled embryos. Small cell populations in the ectoderm of embryos at different developmental stages were labelled with DiI and DiO in the positions indicated in the diagrams (A″–F″). The embryos were grown until stages 15–18 when both the nasal and lens placode can be identified by their morphology. (A, A′, A″) A cell population labelled at stage 7 just outside the neural plate (A″) gave rise to progeny in both the lens and the nasal placode (A, A′). (B, B′, B″) Two cell populations in a two-somite embryo were labelled at the same anteroposterior level, but in different mediolateral positions (B″). Both labels contributed to the olfactory placode (B, B′) and surface ectoderm, while only DiI-labelled cells (red) gave rise to lens cells. (C, C′, C″) Groups of cells in a five-somite embryo were labelled at different anteroposterior levels (C″). Both DiI (red)- and DiO (green)-labelled cells populated the lens (C, C′) as well as the nasal placode; DiO-labelled cells are also found in surface ectoderm. (D, D′, D″) Two cell populations were labelled at the seven-somite stage (D″). DiI-labelled cells gave rise to the lens and surface ectoderm (red; D, D′), while DiO-labelled cells contributed to the nasal placode and the adjacent ectoderm (green; D). Note: DiO label in the eye is confined to optic vesicle derived cells and is due to accidental labelling of the vesicle underlying the surface ectoderm. (E, E′, E″) When labelled at the nine-somite stage cells in the anterior ectoderm give rise to the olfactory placode and surrounding ectoderm (E, E″). F, F′ and F″ show an embryo labelled in the ventral ectoderm at the 11-somite stage (ventral view in F″). DiO-labelled cells close to the anterior neuropore contributed to the olfactory placode (F, F′), while DiI-labelled cells further away populated the ectoderm of the first branchial arch (F). A′, B′, E′ and F′ show sections through the nasal placode of the embryos shown in A, B, E and F, respectively; C′ and D′ represent sections through the lens of the embryos shown in C and D, respectively.

Lens and olfactory precursors arise from a common domain

At stage HH6–7, olfactory and lens precursors reside in the anterior ectoderm next to the neural plate where they are intermingled with future epidermal cells (Fig. 3). In addition, precursors for all three cell populations are found amongst prospective neural cells in the lateral edge of the neural plate (width: 10–15% ml-np). Nasal precursors spread from the most anterior tip of the neural plate to approximately one third of its length (0–28% pp-hn), while future lens cells are found slightly more posterior (8–36% pp-hn). Precursors for both placodes reach out into the lateral epiblast as far as 50% of the width of half the neural plate (50% ml-np).

At the two- to three-somite stage ($HH7^{+}/8^{-}$), the anterior neural folds contain precursors for all four tissues: nasal and lens placode, neural tube and epidermis (Fig. 3). Nasal and lens precursors are found from the most anterior tip (0% anr-som) to about one third of the distance between the first somite and the anterior neural ridge (30% anr-som). The adjacent ectoderm contains a mixture of future olfactory, lens and epidermal cells. While many cell groups contributed progeny to more than one tissue (51/121; nasal and

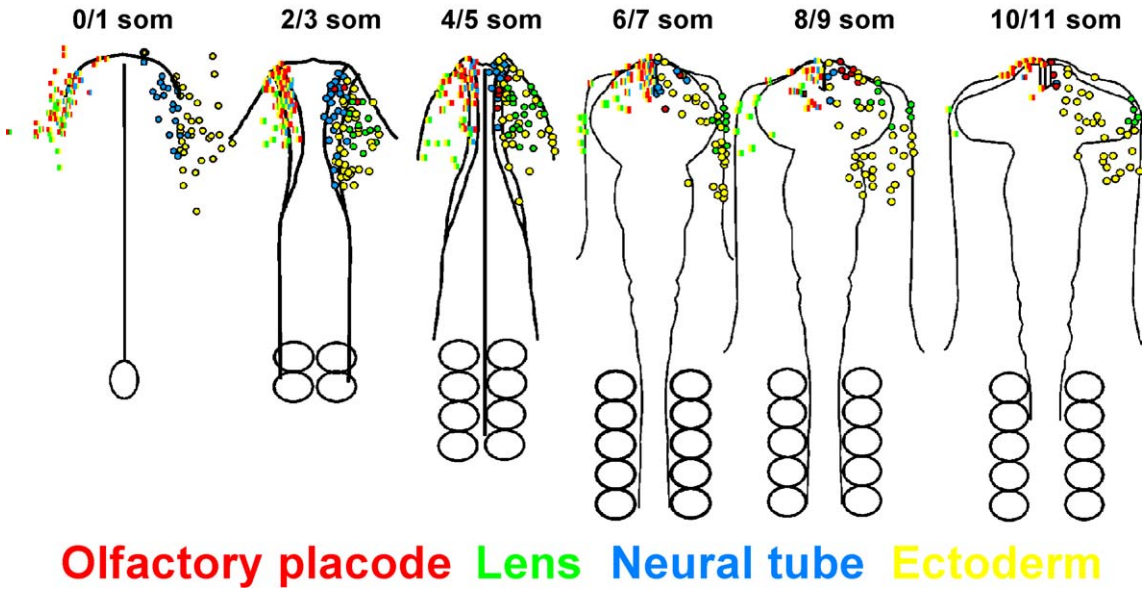

Fig. 3. Fate map of lens and olfactory precursors between stages HH6 and 10. Small cell populations in the epiblast were labelled with DiI and DiO at stages HH6–10; embryos were grown until the nasal and lens placodes were morphologically visible. Each circle (right) or square (left) represents one dye injection; each injection contributed to one (circle) or more tissues (squares) that are colour coded; red: olfactory placode, green: lens placode, blue: neural tube, yellow: surface ectoderm (including corneal ectoderm and ectoderm of the branchial arches). At stage HH6/7 (0–1 somites), lens and nasal placode precursors are intermingled with each other and future epidermis cells; there is some overlap with neural precursors in the outer edge of the neural plate. At the two- to three-somite stage ($HH7^{+}/8^{-}$), precursors for both placodes localise to the anterior neural folds and the adjacent ectoderm and are still mixed. From the four- to five-somite stage onwards ($HH8/8^{+}$), future lens and nasal cells begin to separate until by the 10-somite stage (HH10), no overlap between both cell groups is observed. At this stage, single injections still contribute progeny to the nasal placode and epidermis or to the lens and epidermis.

lens, nasal and epidermal, lens and epidermal, or all three), very few injections led to labelled cells in both the brain and the olfactory placode (6/51) and none simultaneously contributed labelled cells to the lens and the central nervous system.

Thus, precursors for both the olfactory and lens placode overlap in a large region of the epiblast next to the anterior neural plate at head fold stages and continue to do so in the anterior neural folds and the adjacent ectoderm until early somite stages. Cells just inside the neural folds occasionally contribute to olfactory placodes, but the majority contributes to the neural tube only.

From stage HH8 onwards, prospective nasal cells begin to accumulate in the anterior neural folds and adjacent ectoderm, while lens precursors concentrate in the lateral ectoderm that will come to overlie the optic vesicle. At this stage, very few dye injections contributed cells to both placodes. Over the next few stages, the separation of lens and nasal precursors continues until it is complete at the 10-somite stage (HH10): prospective lens cells are located in the ectoderm adjacent and dorsal to the optic vesicles and presumptive nasal cells have converged to the most anterior ectoderm surrounding the open neuropore.

Extensive cell movements lead to the segregation of lens and nasal precursors

Our fate map analysis shows that lens and olfactory precursors originally arise from a common domain shared by other ectodermal derivates. This raises the intriguing question of how these cells become segregated over time. One possibility is that cells divide and move randomly with no predisposition to a particular ectodermal fate; those that end up close to the anterior neural tube receive signals instructing them to differentiate into olfactory placode, while those that localise next to the optic vesicle are induced to become lens. Alternatively, the two sets of precursors may already differ before they start to migrate and then move in a directed manner to their appropriate locations in the presumptive olfactory and lens domains.

To begin to distinguish between these possibilities, we performed a time-lapse analysis of embryos from stage 7 or 8, which were then filmed for 12–14 h until they reached stages 10–11. We analysed 28 embryos, each with multiple DiI injections on the left and right side within the common olfactory-lens precursor domain as well as more caudally to facilitate comparison between different regions.

In all cases, DiI-labelled cells moved extensively. Most cells tended to move from lateral to medial towards the midline as the neural folds closed (Fig. 4A: $t = 300$ min and $t = 375$ min; white arrowheads; Fig. 4B: $t = 140$ min; red arrowhead). However, some labelled cells subsequently underwent extensive lateral movements, generally directed towards the future lens territory at about stage $HH8^{+}/9$ (Figs. 4A, B, green arrowheads). Analysis of the trajectories of cell groups (Figs. 4B and 5) showed that after initially following a common track, individual cell populations often

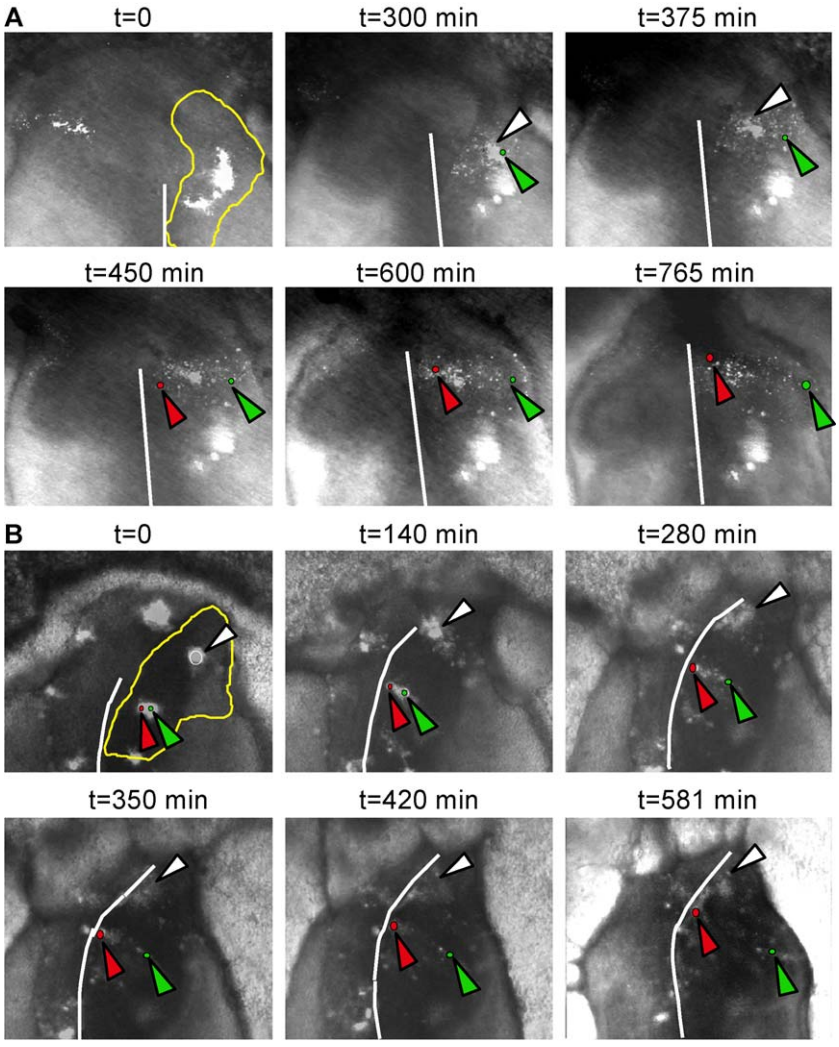

Fig. 4. Lens and olfactory precursors show directional movement to their final target position. Single frames from time-lapse movies illustrate that extensive movements occur within the placode domain, leading to the segregation of olfactory and lens precursors. Times are indicated above each frame in minutes and the white line indicates the embryonic midline. (A) An embryo that received multiple DiI injections within the placode domain (indicated by yellow outline) at stage HH8^{-}. The green cell group (green arrowhead) underwent lateral movement towards the lens whereas the adjacent cells (white arrowhead) moved rostromedially towards the olfactory placode. The group indicated in red (red arrowhead) first came into the plane of focus near the midline at t = 450 min. From this location, this cell population moved rostrally to the olfactory placode. (B) An embryo labelled at stage HH8, with three injection sites within the placode domain (yellow outline). The green cell group (green arrowhead) moved laterally towards the lens. The immediately adjacent red population (red arrowhead) moved medially in the direction of the olfactory placode. The most rostral injection (white circle and arrowhead) underwent little apparent cell movement and cells became localised to the anterior neural folds within the olfactory placode domain.

split such that one group moved more medially and the other laterally away from the midline.

Those cells that remained medially often turned, at times abruptly, and began moving rostrally towards the presumptive olfactory placode (Fig. 4B, red dots and arrowheads). Even from quite disparate injection sites at several different rostrocaudal levels, we observed cells merging at the anterior tip of the embryo. This suggests that at least some cells may move in a directional fashion towards the presumptive olfactory placode, while others move towards the future lens. However, other cells (e.g. those derived from the most rostral injections) underwent little cell movement before becoming localised in the olfactory territory (Fig. 4B, white arrowheads).

Early co-localisation of Pax6 and Dlx5 mRNA defines a common nasal-lens territory, later separation of the Pax6 and Dlx proteins correlates with acquisition of placodal identity

In *Drosophila*, the homeobox transcription factor Dll and the paired domain protein Ey are initially co-expressed in the eye-antennal disc; during the second larval instar, however, a negative feedback loop acts to restrict Dll to the antennal and Ey to the eye primordium and establishes disc identity (Cohen et al., 1989; Dong et al., 2000; Halder et al., 1995; Kurata et al., 2000; Panganiban and Rubenstein, 2002; Quiring et al., 1994; Sunkel and Whittle, 1987). To test whether a similar molecular mechanism might regulate

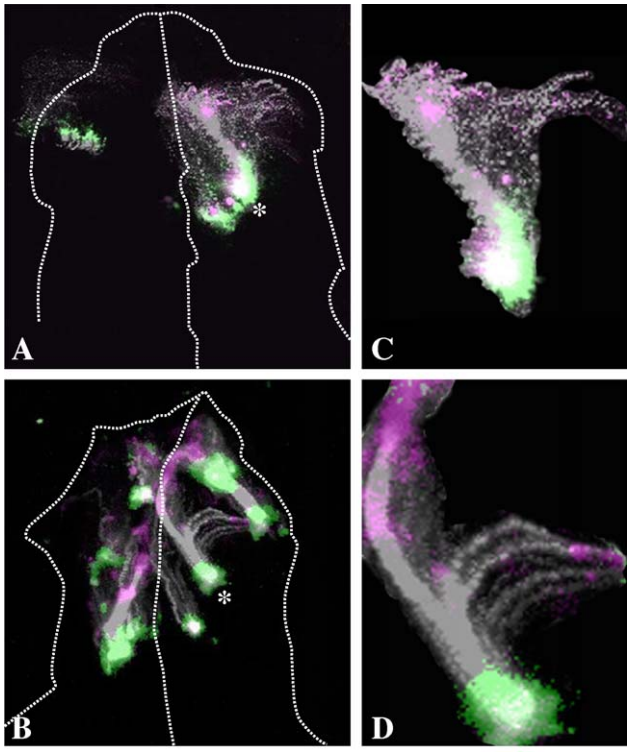

Fig. 5. Individual cell populations split into streams of cells moving towards different targets. (A and B) Trajectories of cell groups shown in Figs. 6A and B, respectively, obtained by collapsing all time frames for each movie into a single image. The first frame is indicated in green and the last in magenta to indicate the start and final position of the label. The outline and midline of the embryo in the last frame are depicted by the dotted lines. C and D show a higher magnification of individual groups (white asterisks in A and B, respectively). Approximately 40 cells were labelled in C and about 20 cells in D.

the segregation behaviour of lens and olfactory precursors observed in the time-lapse analysis, we investigated the expression of *Dlx5* and *Pax6* transcripts and of the proteins they encode in relation to the separation of future lens and nasal cells.

The mRNAs of both genes overlap at the one- to two-somite stage (HH7) in the anterior ectoderm (Figs. 6A, A′, D, D′), although expression appears to be mosaic with some cells expressing high and others low levels of transcripts (Figs. 6A′, D′ insets). Like the lens and olfactory precursors, the *Pax6* domain surrounds the neural plate from its most anterior tip to about 35% of its length, while *Dlx5* expression continues more posteriorly, to the level of Hensen's node. Both transcripts extend mediolaterally from the edge of the neural plate into the ectoderm for about 50% of the width of half the neural plate. From stage HH8 onwards, the two expression domains begin to separate: while *Dlx5* transcripts concentrate in the most anterior neural folds and ectoderm, *Pax6* remains expressed strongly in the neural folds and the more posterior surface ectoderm (Figs. 6B, E). By stage 10, *Dlx5* expression is confined to the most anterior tip of the surface ectoderm (Figs. 6F, F′), where *Pax6* is absent (Figs. 6C, C′). Thus, the region where *Dlx5* and *Pax6* mRNAs are co-expressed matches precisely the position where lens and nasal precursors reside at early stages, and the expression domains separate as cells begin to segregate, suggesting that the mechanisms that regulate differential transcription of these genes are initially deployed as the two streams of cells separate.

However, a slightly different result is obtained when examining the distribution of Pax6 and Dlx proteins. At HH7, the level of expression of both proteins is extremely low (not shown) becoming robust at HH8, when all cells in the nasal-lens territory show high levels of Dlx and Pax6 (Figs. 7A–C). Unlike the mRNA, Dlx protein is maintained in presumptive lens cells until stage HH12/13 to disappear from lens cells as soon as placode morphology is established. The lens only contain $Pax6^{+}/Dlx^{-}$ cells (Figs. 7G–H). Cornea precursors overlying the lens are mainly $Pax6^{+}/Dlx^{-}$ except for few double-labelled cells in the periphery. In contrast, the anterior ectoderm containing olfactory precursors retains Dlx protein, while losing Pax6 around stage HH10 (not shown) and is clearly $Pax6^{-}$ once the placode is formed (Fig. 7H). Thus, rather than reflecting the separation of olfactory and lens precursors (like the mRNA), the differential expression of Dlx and Pax6 proteins correlates with the acquisition of a particular fate (e.g. lens) and placodal morphology.

Persistent expression of Dlx5 regulates cell sorting

In the fly, Dll and Ey have been suggested to negatively regulate each other to determine antennal vs. eye disc identity (Kurata et al., 2000). To test whether their vertebrate homologues have similar functions during nasal and lens placode development, we maintained expression of Dlx5 in lens precursors beyond the time when they have normally lost it and investigated the effect on their differentiation, localisation or fate. If vertebrate placode development uses a molecular mechanism akin to the one that operates in *Drosophila* imaginal disc formation, this would predict that $Dlx5^{+}$ future lens cells should lose their lens character and lens-specific gene expression.

The ectoderm containing lens precursors of HH8–10 chick embryos was transfected by electroporation with GFP control (pCAB-IRES-GFP) or Dlx5 vector (pCAB-Dlx5-IRES-GFP). To investigate the behaviour of cells during the entire process of lens formation, embryos were grown for different times to reach early lens placode or later lens vesicle stages (HH12–20). The location of electroporated cells was monitored in whole mounts by their GFP expression. Numerous GFP^{+} cells were found within the lens of all control electroporated embryos (16/16; Figs. 8A, C, D). In contrast, while $Dlx5^{+}/GFP^{+}$ cells were abundant in the ectoderm overlying the lens (future cornea), head epidermis and olfactory placodes, no $Dlx5^{+}/GFP^{+}$ cells contributed to the lens itself in Dlx5 electroporated embryos (2/23; Figs. 8E, G, H). Most of the experimental embryos (22/23) showed either extremely small or deformed lenses whereas

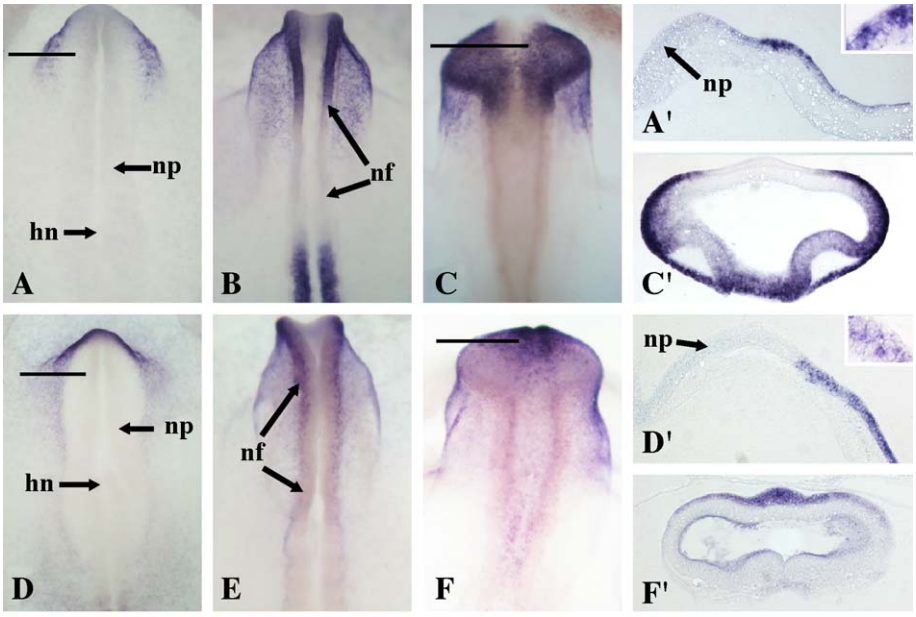

Fig. 6. Changes in *Dlx5* and *Pax6* expression reflect the spatial arrangement of nasal and lens precursors. Whole mount in situ hybridisation showing the expression of *Pax6* (A–C; A′, C′) and *Dlx5* (D–F; D′, F′) at stages 7 (A, D), 8 (B, E) and 10 (C, F). *Pax6* and *Dlx5* are co-expressed at the border of the neural plate at stage 7 (A, A′, D, D′) although expression seems to be mosaic (insets in A′, D′); while *Dlx5* begins to concentrate in the most anterior ectoderm and neural folds (E; stage 8), *Pax6* remains expressed in the folds and the ectoderm lateral to the diencephalic region (B; stage 8). By stage 10, *Pax6* (C; C′) and *Dlx5* (F; F′), expression domains are mutually exclusive. A′, C′, D′ and F′ show sections through the embryos in A, C, D and F, respectively, at the level indicated by black lines. hn: Hensen's node, nf: neural folds, np: neural plate.

controls looked normal. In addition, control electroporated cells were well dispersed within the lens and head ectoderm, whereas Dlx5-expressing cells were always found in clusters indicating that they display different adhesive properties than their neighbours (compare insets in Figs. 8A and E).

To investigate the phenotype with better cellular resolution, all embryos were sectioned and immunostained for GFP and the lens-specific protein δ-crystallin. While both proteins are co-expressed in control electroporated embryos (Figs. 8B, D), none of the $Dlx5^{+}/GFP^{+}$ cells in experimental embryos expressed δ-crystallin (Figs. 8F, H). Occasionally, a single isolated $Dlx5^{+}/GFP^{+}$ cell was present in the lens; however, these cells have lost lens morphology as well as δ-crystallin expression (not shown). Sections of embryos at stage HH12/13 revealed that $Dlx5^{+}/GFP^{+}$ cells are excluded from the lens placode as soon as the cells develop the typical columnar morphology (not shown). The sections also confirmed that lenses in Dlx5 electroporated embryos display abnormal morphology and are generally much smaller than lenses in control embryos or on the contralateral side. Consequently, optic vesicle formation was often severely disrupted (Figs. 8F, G). Thus, Dlx5-expressing cells are never incorporated into the lens placode but are excluded from it as soon as it forms, suggesting that down-regulation of Dlx5 is an important prerequisite for cells to adopt a lens fate. Furthermore, these results indicate that by the time the proteins are differentially expressed, the transcription factors Dlx5 and Pax6 may regulate cell sorting events to ensure that cells with the incorrect expression profile do not end up in inappropriate placodes.

Discussion

A common territory for lens and olfactory precursors

In a 3-day-old chick embryo, the olfactory and lens placodes are clearly separate entities adjacent to the ventral forebrain and the optic vesicle, respectively. Here, we report that at earlier stages, precursors for both structures are extensively mixed and occupy a common domain surrounding the anterior neural plate at head fold stages and only begin to separate at early somite stages. Therefore, this finding differs from earlier fate and specification maps (Carpenter, 1937; Kozlowski et al., 1997; Röhlich, 1931; Rudnick, 1944) from amphibians, fish and amniotes reporting an early segregation of lens and nasal cells into distinct domains. In zebrafish, in agreement with our findings, future olfactory cells converge from a large field towards their final position in the placode (Whitlock and Westerfield, 2000). Although their co-localisation with lens precursors has not been reported, the possibility that there is considerable overlap at earlier stages is not excluded.

It has been suggested that olfactory precursors arise from the neural territory either from isolated cell groups that migrate away from the neural plate (Farbman, 1992; Verwoerd and van Oostrom, 1979) or from a large territory surrounding the future telencephalon (Whitlock and Westerfield, 2000). Similarly, an earlier fate map using chick-quail chimaeras localised the presumptive olfactory placode to a very small domain within the neural folds at the three- to four-somite stage (Couly and Le Douarin, 1985, 1987). In

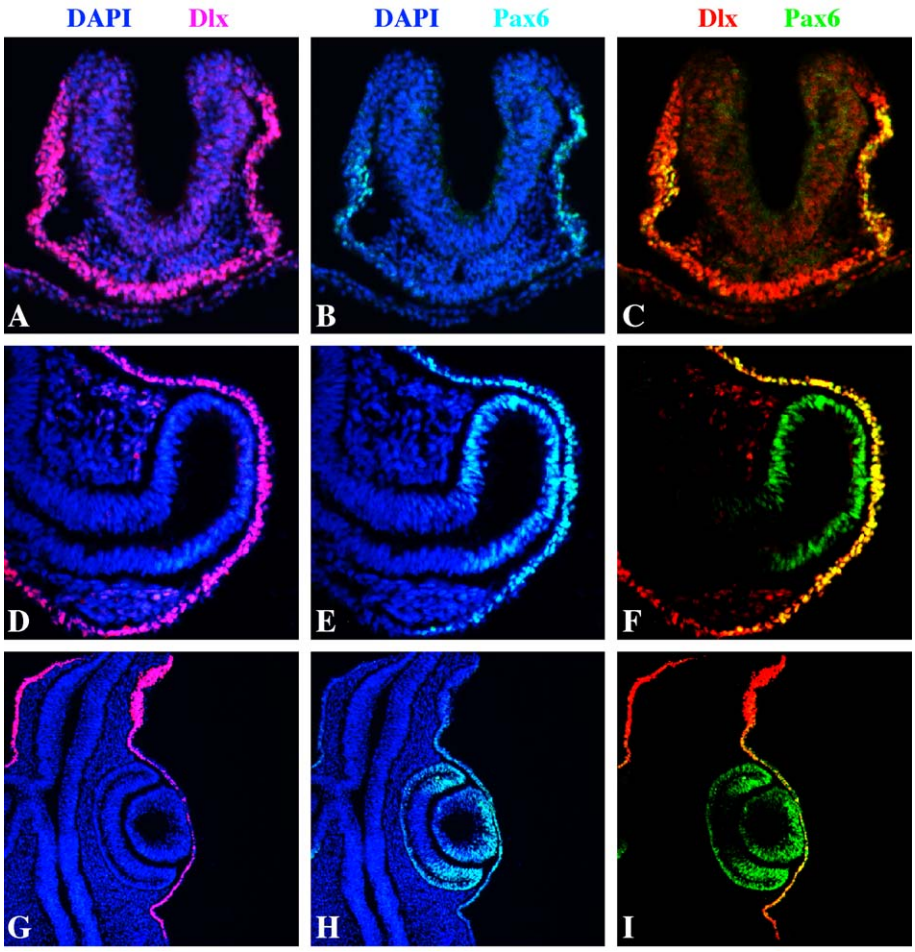

Fig. 7. Dlx and Pax6 proteins are differentially expressed at the time of placode formation. Immunohistochemistry was performed on frozen sections of stages HH8 (A–C), 12 (D–F) and 15 (G–I) embryos using a pan-Dlx (red; A, D, G, C, F, I) and a Pax6-specific antibody (green; B, E, H, C, F, I). To visualise nuclei, sections were stained with DAPI (blue). C, F and I show overlay of Dlx and Pax6 staining. At stage 8, Dlx (A) and Pax6 (B) are co-expressed in the lateral ectoderm next to the anterior neural plate (C, yellow) and continue to do so in the presumptive lens ectoderm until stage 12, just before the lens placode forms (D–F). Note: optic vesicle is $Pax6^{+}$ (green). The mature lens placode (G–I) has lost Dlx expression (G, I), but retained Pax6 (H, I); likewise, the future cornea gradually loses Dlx protein. In contrast, the olfactory placode is strongly Dlx positive, but does not show any Pax6 expression.

contrast, our data show that the majority of nasal precursors arise from the non-neural ectoderm in close association with future lens cells. In fact, while single injections into the neural folds at early somite stages often contribute to the olfactory and lens placode or to these placodes and surface ectoderm, only a negligible number of labelled cell groups give rise to progeny in both the neural tube and the nasal placode. Even at earlier stages, there is only limited mixing of future neural, lens and olfactory cells. Therefore, the segregation of neural from olfactory and lens progenitors is almost complete by early somite stages.

Genetic evidence suggests that not only do nasal and lens precursors share a common origin, but they also may use similar molecular pathways for their initial specification (Grindley et al., 1995; Quinn et al., 1996; van Heyningen and Williamson, 2002; Walther and Gruss, 1991; Wawersik et al., 2000; Xu et al., 1997). *Pax6* expression clearly matches the nasal-lens territory at head fold stages. Mice lacking Pax6 function never form nasal or lens placodes (Grindley et al., 1995), suggesting that Pax6 is required at early stages of their development, perhaps at the time when the common territory is specified. Afterwards, the molecular events that control differentiation of each placode seem to diverge. While the lens maintains *Pax6* expression throughout development, olfactory precursors lose *Pax6* at intermediate stages before re-acquiring it at late placode stages.

Specification of lens and olfactory precursors parallels the formation of the eye-antennal imaginal disc of Drosophila

Like the nasal epithelium in vertebrates, the insect antenna contains olfactory receptor cells responsible for odour discrimination. The Dlx-protein Dll and the Pax6 homologue Ey are initially co-expressed in the eye-antenna disc; however, by the time the eye and antennal primordia become distinct, Ey expression is restricted to the eye, while

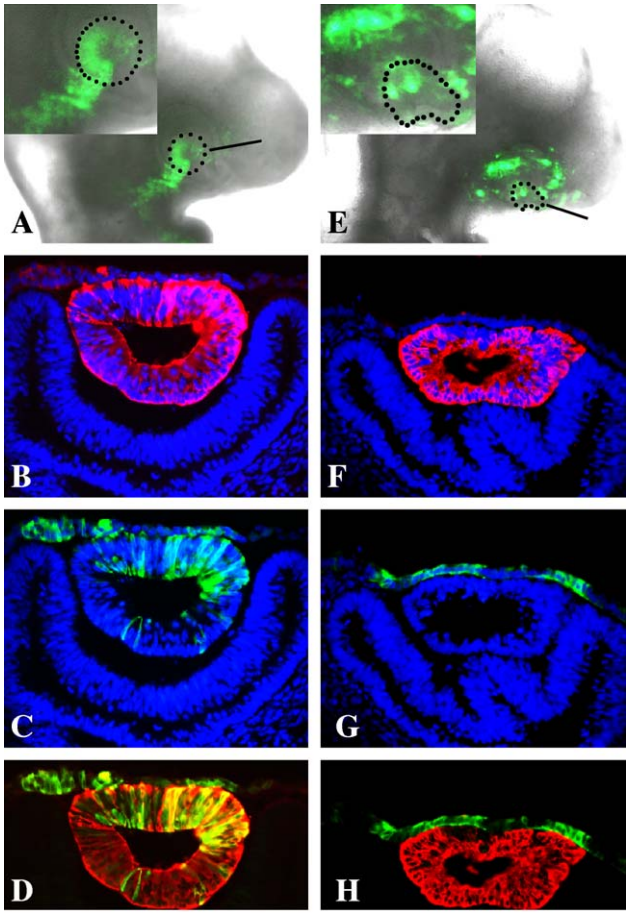

Fig. 8. Lens cells that continue to express Dlx5 lose lens character and are excluded from the lens. The lens-olfactory territory was electroporated with pCAB-IRES-GFP (control; A–D) or pCAB-Dlx5-IRES-GFP (E–H) at stage HH8–9. The distribution of GFP positive cells was evaluated in whole mounts (A, E) and cryosections (C, D, G, H). Control electroporated cells are found in the lens as well as widespread in the head ectoderm (A, C, D, inset in A), while Dlx5-containing cells never occupy the lens and cluster (E, G, H, inset in E). Cryosections were stained using antibodies against GFP (C, D, G, H; green) and the lens-specific δ-crystallin (B, D, F, H; red) and DAPI to visualise nuclei (blue). D and H show overlays of GFP and δ-crystallin expression. In control embryos, lens morphology is normal and GFP and δ-crystallin are co-expressed in the lens (B, C; yellow in D). In contrast, in Dlx5 electroporated embryos the lens is smaller and malformed and no Dlx5-expressing cells (green, G, H) are found in the δ-crystallin positive lens (F, H).

Dll is only found in the antennal anlage (Kumar and Moses, 2001a,b). Indeed, these transcription factors negatively regulate each other (Kurata et al., 2000) and are required to establish eye and antennal identity, respectively (Cohen et al., 1989; Dong et al., 2000; Halder et al., 1995; Panganiban and Rubenstein, 2002; Quiring et al., 1994; Sunkel and Whittle, 1987; for review: Gehring, 1996; Kumar and Moses, 2001c). In the chick, the situation is comparable: the co-expression of *Dlx5* and *Pax6* transcripts precisely matches the common lens-olfactory territory. Their expression domains separate just as lens and nasal precursors segregate, with *Dlx5* concentrating nasally and *Pax6* accumulating in the lens.

Based on these observations, an intriguing possibility is that these two factors control the process of segregation as well as the directional movements observed in films. However, analysis of Dlx and Pax6 proteins reveals that both factors remain co-expressed for much longer and Dlx is only lost from lens cells when the placode is established as a morphological entity, suggesting that these factors are unlikely to confer placodal identity until the time of placode formation. In agreement with this, maintenance of Dlx5 protein in future lens cells results in the loss of lens morphology and lens-specific gene expression, and the cells themselves fail to incorporate in the forming lens. Interestingly, in a complementary experiment, $Pax6^{-/-}$ cells in mouse chimaeras sort out from neighbouring wild-type lens cells (Collinson et al., 2000). Together, these observations suggest that the loss of Dlx protein is essential for lens cells to acquire lens identity, reminiscent of how eye and antennal disc identity is conferred in the fly.

Do Pax6 and Dlx5 regulate cell sorting at placode stages?

The observations that lens cells that are forced to maintain Dlx5 expression (this study), as well as $Pax6^{-/-}$ cells in mouse chimaeras (Collinson et al., 2000) are expelled from the developing lens, indicate that at the time of placode formation, Pax6 and Dlx transcription factors regulate a cell sorting event to ensure that only cells with appropriate fates are included in the lens. Indeed, Pax6 is known to regulate cell adhesive properties (Chalepakis et al., 1994; Davis et al., 2003; Stoykova et al., 1997; Tyas et al., 2003). Similarly, in the leg imaginal disc in *Drosophila*, $Dll^{-/-}$ clones segregate from their Dll-positive neighbours (Gorfinkiel et al., 1997; Panganiban and Rubenstein, 2002; Wu and Cohen, 1999). Thus, it is likely that the control of adhesive properties by these transcription factors is important for placode formation.

Extensive cell movements as a general feature of placode development

Our findings reveal that extensive cell movements accompany the formation of both the lens and olfactory placode in the chick embryo. In addition, individual cells or cell groups constantly change their neighbours until a homogeneous domain of presumptive lens or olfactory cells is formed. Similar movements and cell rearrangements have recently been described during the formation of the chick otic (Streit, 2002) and of the zebrafish olfactory placode (Whitlock and Westerfield, 2000), raising the possibility that this is a general feature of placode formation.

From within a large pre-placodal domain next to the anterior neural plate, precursors for specific placodes converge to their final position while undergoing constant cell rearrangements. How do these cells segregate? One possibility is that cell movements are random and that only cells that happen to encounter appropriate inducing signals

are directed towards a specific fate. An alternative is that cells move directionally to their final destination and/or sort out from their neighbours due to differential properties. The findings that some cells like lens (this study) and otic precursors (Streit, 2002) seem to move against the mainstream (laterally away from the neural tube and midline) suggests that directional cues may govern their behaviour.

As discussed above, Pax6 and Dlx5 appear to act late, perhaps in connection with the acquisition of specific fates, rather than early to control the segregation behaviour of lens and nasal precursors. On the other hand, in situ hybridisation reveals mosaic expression and more importantly an early bias of mRNA distribution for both factors at the time when streams of future lens and olfactory cells start to diverge, suggesting that the upstream regulatory mechanisms that govern both migration and the differential expression of these factors are deployed very early. Taken together, our study suggests that cells within the common placodal territory may have an early bias towards a specific placodal fate, which is subsequently reinforced by a combination of two mechanisms: local signals to control gene expression, including which factor wins over the other in a tug-of-war (as in the fly), and cell sorting mechanisms, which leads to incorporation into appropriate placodal territories.

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